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Insights into the role of latent virus in the vasculitis in sheep-associated malignant catarrhal fever

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Insights into the role of latent virus in the vasculitis in sheep-associated malignant catarrhal fever

Malignant catarrhal fever (MCF) is a generally fatal γ-herpesvirus-associated sporadic disease in ruminants. Systemic vasculitis, mainly in the form of a mononuclear arteritis, is a key pathological process, and cytotoxic T cells play a major role in it. So far, the pathogenesis of MCF vasculitis is not clear. We hypothesized that it develops due to interactions between virus infected cells and immune cells and undertook a retrospective *in situ* study on rete mirabile arteries of ovine herpesvirus-2 associated MCF cases.

The results indicate that the arteritis develops from an adventitial infiltration, with superimposed luminal recruitment of leukocytes. Infiltration of the wall can then lead to chronic proliferative changes. Besides T cells, macrophages are the dominant infiltrating cells, both proliferate locally. RNA-*in situ* hybridisation and immunohistology served to show that the process is associated with widespread latent viral infection, not only in infiltrating leukocytes, but also in endothelial cells, medial smooth muscle cells and adventitial fibroblasts.

This suggests that latently infected, locally proliferating T cells and macrophages are equally important for the vasculitis in MCF. The initial trigger/insult is not yet known, but there is evidence that, among others, latently infected, acvitated endothelial cells play a role in this. Releasing pro-inflammatory mediators, activated monocytes/macrophages could then induce the characteristic vascular changes.

Keywords: cattle, malignant catarrhal fever, vasculitis, rete mirabile

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Einblicke in die Rolle vom latenten Virus in der Vaskulitis des ovinen-assoziierten bösartigen Katarrhalfiebers

Das Bösartige Katarrhalfieber (BKF) ist eine in der Regel tödliche γ-Herpesvirus-assoziierte sporadische Erkrankung bei Wiederkäuern. Schlüsselprozess ist eine systemische Vaskulitis, v.a. als mononukleäre Arteritis, zytotoxische T-Zellen spielen eine wichtige Rolle. Die Pathogenese der Vaskulitis ist unklar. Die vorgelegte retrospektive Studie folgt der Hypothese, dass deren Entwicklung auf Interaktionen zwischen Virus-infizierten Zellen und Immunzellen basiert; sie untersuchte die Arterien des Rete mirabile in OvHV-2-assoziierten BKF-Fällen.
Die Ergebnisse deuten an, dass die Arteritis Folge einer primär adventitiellen, nachfolgend auch luminalen Leukozytenrekrutierung ist. Die Infiltration der Wand kann dann zu chronisch-proliferativen Prozessen führen, sie wird neben T-Zellen von Makrophagen dominiert; beide Zelltypen proliferieren lokal. Mittels RNA-*in situ*-Hybridisierung und Immunohistologie konnte gezeigt werden, dass der Prozess mit einer latenten Virusinfektion der infiltrierenden Leukozyten sowie von Endothelzellen, glatten Muskelzellen der Media und Fibroblasten der Adventitia verbunden ist.
Latent infizierte T-Zellen und Makrophagen scheinen demnach von vergleichbarer Relevanz für die BKF-Vaskulitis zu sein. Deren Auslöser ist noch nicht bekannt, jedoch scheinen u.a. aktivierte Endothelzellen eine Rolle zu spielen. Aktivierte, pro-inflammatorische Mediatoren freisetzende Monozyten/Makrophagen könnten dann die characteristischen Gefässveränderungen bewirken.

Keywords: cattle, malignant catarrhal fever, vasculitis, rete mirabile

Insights into the role of latent virus in the vasculitis in sheep-associated malignant catarrhal fever

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Abstract

Malignant catarrhal fever (MCF) is a generally fatal γ-herpesvirus-associated sporadic disease in susceptible dead-end hosts in which a systemic vasculitis is a key pathological process. The vasculitis generally presents as a mononuclear arteritis in which cytotoxic T cells which have recently been shown to carry productive viral infection, play a major role. Nonetheless, the pathogenesis of MCF vasculitis is not yet clear. We hypothesized that it develops due to an interaction between virus infected cells and immune cells and undertook a retrospective in situ study on the rete mirabile arteries of confirmed ovine herpesvirus-2 associated MCF cases.

Our results indicate that the arteritis develops from an adventitial infiltration upon which luminal recruitment of leukocytes is often superimposed; infiltration of the wall can then lead to chronic changes including neointimal proliferation. Macrophages are the dominant infiltrating cells besides T cells, and proliferate locally. Using RNA-*in situ* hybridization and immunohistology, we could show that the process is accompanied by widespread latent viral infection, not only in infiltrating leukocytes, but also in vascular endothelial cells, medial smooth muscle cells and adventitial fibroblasts.

Our results suggest that latently infected T cells, monocytes and locally proliferating macrophages are equally important for the vasculitis in MCF. The initial trigger/insult that leads to leukocyte recruitment and activation is not yet known, but there is at least evidence that latently infected, acvitated endothelial cells play a role in this. Activated monocytes/macrophages would then release the necessary pro-inflammatory mediators and, eventually, induce the characteristic vascular changes.

Keywords: cattle, malignant catarrhal fever, vasculitis, rete mirabile

Malignant catarrhal fever (MCF) is a generally fatal γ-herpesvirus-associated disease with a wide range of susceptible hosts, affecting ungulates of the order Artiodactyla including cattle, water buffalo, bison, deer and other wild ruminants like antelope, elk and reindeer.[29,37,45,56]. The disease is occasionally seen also in pigs,[3,32] and can be experimentally induced in various additional species, such as rabbits, guinea pigs, and hamsters.[5,7,8,13,21]

The causative agents of MCF are the so-called MCF viruses, herpesviruses of the subfamily *Gammaherpesvirinae*, genus Macavirus, of which at least six are pathogenic under natural conditions. Among these, ovine herpesvirus 2 (OvHV-2) is the most relevant globally. It is endemic in sheep and causes the so-called sheep-associated MCF (SA-MCF) in various ruminant species;[27] it is also the virus reported in association with MCF outbreaks in pigs.[3,32] Alcelaphine Herpesvirus 1 (AlHV-1) is endemic in wildebeest and is also carried by goats; it causes wildebeest-associated MCF (WA-MCF) in domestic cattle.[45] The other MCF viruses are less common. Caprine Herpesvirus- 2 (CpHV-2) is endemic in domestic goats and has been reported in MCF in two deer species and in a domestic water buffalo in Switzerland.[10,12,23,25] White tailed deer and red brocket deer are susceptible to the MCFV-WTD (malignant catarrhal fever virus – white-tailed deer), which is carried by domestic goats, Ibex MCF virus (Ibex-MCFV) has been identified in bongo and anoa with MCF and is carried by the nubian ibex,[17] and Alcelaphine Herpesvirus 2 (AlHV-2) is carried by hartebeest and topi and has been reported as a cause of MCF in barbary red deer and experimentally in bison.[24,29]

MCF viruses are hosted in the above listed reservoir species (so-called “host species”). As γ-herpesviruses, they have evolved with their host species, where infection is, in the majority of cases, not associated with disease, though recent studies, based on natural cases and experimental infection have confirmed that

sheep can develop OvHV-2 associated MCF.[28,50,51] Lambs usually become infected soon after birth, via horizontal transmission.[2,56] Natural transmission studies in sheep suggested that white blood cells are the first cells to carry infectious virus after infection,[20] experimental infection studies identified lytically infected lower airway epithelial cells as the main source of infectious virus early (7 days) after infection.[66] OvHV-2 infected healthy lambs shed infectious virus intermittently, mainly through nasal secretions, but also via the alimentary and urogenital tracts.[15,20,26] Infected animals generally develop a persistent latent infection, with white blood cells as the reservoir.[19,67] Further studies identified the infected leukocytes as CD4+ and CD8+ T cells.[36,58] Viral reactivation can then result in transmission to susceptible species.[37,56] When MCF viruses are horizontally transmitted to susceptible hosts, in natural infections of cattle an incubation period of 3 to 8 weeks has been suggested.[52] A generally fatal multisystemic disease can develop, characterized by lymphoid proliferation and infiltration with widespread vascular and epithelial lesions that can result in different clinical forms.[30,52,64,68] A feature of MCF at least in cattle is that the majority of outbreaks are sporadic with mainly single affected animals;[56] however, serious outbreaks have been reported in cattle and, more often, in certain species of deer and in bison.[1,6,53] The latter appear to be particularly susceptible to infection, which is reflected in higher numbers and rapid death of affected animals.[6,44,45] Susceptible hosts do not shed the virus, at which point the viral cycle terminates.[68] It is believed that in these, disease is the result of direct virus-cell interactions or a dysregulated immune-mediated response against infected cells.[61]

The diagnosis of MCF is based on clinical signs, histopathological features and the detection of viral DNA by PCR.[56] In affected species, the main gross findings are erosions and ulcerations in upper respiratory tract and gastrointestinal tract, keratoconjunctivitis, and lymphadenomegaly.[45,56,68]. Histological examinations show

that the mucosal lesions, i.e. the necrosis of the respiratory, alimentary and urinary epithelium are due to a systemic vasculitis which also involves vessels in other tissues, such as the brain.[30,45] The vasculitis is thought to be the consequence of immune dysregulation that results in accumulation of lymphocytes, predominantly CD8+ T cells, with natural killer cell-like properties.[39,40,56,61]

It has been shown that the viral load increases slowly in the early phases of the disease; after clinical disease outbreak, high viral loads are measured.[64] This could be the result of high viral replication or of replicating, virus infected cytotoxic T cells which can in turn lead to the characteristic lymphocytic hyperplasia.[35,64,68]

The above-mentioned systemic vasculitis is a key pathological process of MCF in all affected species. Arteries are predominantly affected, exhibiting a mononuclear infiltration of media and adventitia. In addition, myocyte necrosis in the tunica media, endothelial cell hyperplasia, intimal thickening and necrotizing vasculitis have been described.[37,45,52,53,68] The most in-depth description of MCF vasculitis results from an ultrastructural study undertaken on rete mirabile arteries of experimentally infected calves. It identified the infiltrating mononuclear cells as lymphocytes and lymphoblasts, with fewer monocytes and macrophages.[30] Later immunohistological examinations showed a dominance of T cells, with abundant activated cytotoxic T cells (CD8+/perforin+ γδ T cells) and fewer CD4+/perforin- αβ T cells, and a substantial proportion of macrophages, whereas the numbers of other leukocytes were negligible.[39,40] A recent study using RNA-in situ hybridization confirmed productive OvHV-2 infection of lymphocytes in the lesions.[49]

The OvHV-2 genome has been cloned and sequenced and the functions of a number of genes assigned. Many of the genes have direct homologues in other herpesviruses whereas some are unique to OvHV-2.[18,65] Like all γ-herpesviruses, OvHV-2 has a productive replication cycle, where most virus genes are expressed

and particles produced as well as a latency where a restricted set of genes are expressed. The genes expressed during latency have been defined and include ORF73 and Ov2.5.[67] The former encodes a nuclear protein (oLANA) that is a homologue of the KSHV LANA protein and the latter (ovIL-10) a functional homologue of cellular IL-10.[4,22]

So far, despite extensive research on the molecular aspects of the disease and its variable clinical presentation, the pathogenesis of MCF, and in particular also the vasculitis is still poorly understood. We hypothesized that the vasculitis develops due to an interaction between virus infected cells and immune cells in the susceptible species. This led us to undertake a retrospective in situ study on MCF vasculitis, making use of confirmed OvHV-2 associated natural MCF cases in cattle. We focused on the arteries in the rete mirabile as these have previously been shown to exhibit changes representative for the vascular lesions also in other organs.[30,45] Our aim was to gain further knowledge on the pathological processes underlying and maintaining the vasculitis in MCF. We therefore investigated the inflammatory and remodeling processes in the context of virus latency using mRNA for ovIL-10 and oLANA protein.

Material and Methods

Animals and tissues

The study was performed on 36 OvHV-2 associated MCF cases (27 cattle of various breeds, 7 buffalos, 2 bisons). Animals ranged from 6 months to 12 years in age (2.67 ± 2.45 years), 33 were female, 3 were male (Supplemental Table S1). Cases had been retrieved from the database (2000–2015) of the Institute of Veterinary

Pathology, Vetsuisse Faculty Zurich, and the Institute of Animal Pathology, Vetsuisse Faculty Berne, where animals had undergone a full diagnostic post mortem examination upon the owners' request.

The diagnosis of MCF and the involvement of OvHV-2 as the causative agent was confirmed on the basis of the gross and histopathological findings, coupled with the detection of OvHV-2 by quantitative PCR (qPCR) on EDTA blood samples (routine diagnostic procedure provided by the Institute of Virology, Vetsuisse Faculty, University of Zurich) prior to necropsy and/or a qPCR (see below) on DNA extracted from formalin-fixed, paraffin embedded tissue specimens as part of the present study. Furthermore, Ov2.5 mRNA (coding for OvHV-2 viral IL-10) and OvHV-2 latency-associated nuclear antigen (oLANA) protein expression was demonstrated within tissue sections of rete mirabile arteries by RNA-ISH and immunohistology, respectively (see below) (Supplemental Table S1).

From all animals, the paraffin blocks containing samples of the rete mirabile were retrieved. The tissue had been fixed in 10% buffered formalin for at least 24 h, followed by trimming and routine paraffin wax embedding.

Quantitative polymerase chain reaction (qPCR) for OvHV-2

The qPCR for OvHV-2 served to confirm the presence of the virus in the diseased animals, using previously reported primer and probe sequences.[63] DNA was extracted from 20 µm thick paraffin sections of lung, lymph nodes, and/or spleen. Briefly, after deparaffination in a series of xylene and ethanol (100%), tissue lysis was performed by proteinase K digestion at 56 °C for 15 – 18 h, followed by incubation in a heating block at 90 °C for 1h to reverse formalin crosslinking. For DNA extraction, the QIAamp DNA FFPE Tissue Kit (QIAGEN, Venlo, Netherlands) was used,

following the manufacturer's instructions. The DNA was stored at -20 °C until further use.

The qPCR was performed on an Applied Biosystems 7500 fast Real-Time PCR system using 96-well plates. The reaction volume (20 µl) consisted of 10 µl of Taqman Mastermix 2x (Thermo Fischer Scientific), each 1 µl of forward and reverse primer (10 µM) and 1 µl of probe (5 µM) for OvHV-2 and 12s-ribosomal DNA, 5 µl of nuclease-free water and 2 µl of template DNA (50 ng/µl). Primers and probes were identical to those in a previous publication.[64] Detection of the 12s-ribosomal DNA internal genome served to normalize for DNA variability and contaminants. Thus, the exact viral copy number was normalized and determined at a detection limit ranging from 2 x 10^7 to 2x 10^0 copies/ 2 µl.

The RT-PCR was run with the following cycling conditions: 1) 20 sec at 95 °C, 2) 3 sec at 95 °C, 3) 30 sec at 56 °C, 40 cycles between steps 2 and 3. The data were collected during step 4 of the RT-PCR programme.

Histology, immunohistology and immunofluorescence

Consecutive sections (4-5 µm) were prepared from the rete mirabile blocks. They were stained with hematoxylin and eosin (HE) and subjected to immunohistology (IH), immunofluorescence (IF) and/or RNA in situ hybridization (RNA-ISH).

IH served to characterize the infiltrating leukocytes, i.e. T cells (CD3+), B cells (CD20+), macrophages (calprotectin+ and/or Iba-1+), to assess cell death (cleaved caspase 3+) and proliferative processes (Ki67+) in the vessel walls, to further assess the muscle layer (α-smooth muscle actin+), and to detect viral antigen in the lesions. For the latter, a custom made polyclonal rabbit antibody against the latency-associated nuclear antigen (oLANA), encoded by the ORF73 of OvHV-2,[4,18] was used. Stains were carried out in a Dako autostainer (Dako, Glostrup, Denmark),

using the horseradish peroxidase method. Antibodies and the respective antigen retrieval and detection methods are listed in Supplemental Table S2.

Briefly, after deparaffinization, antigen retrieval was performed for all antigens except for α-SMA, by incubation of the slides with citrate buffer (pH 6) or EDTA buffer (pH 9) at 98°C for 20 min in a pressure cooker.

After incubation with the primary antibodies for 60 min at room temperature (RT), endogenous peroxidase was blocked by incubation with peroxidase blocking solution (Dako, S2023) for 10 min at RT. This was followed by incubation with the matching secondary antibodies and the appropriate detection kits (Table S2). Sections were washed with phosphate buffered saline (pH 8) between each incubation step. Finally, sections were counterstained with hematoxylin for 20 s and mounted. Consecutive sections, incubated with nonreactive monoclonal or polyclonal antibodies, as well as rete mirabile from uninfected animals (non-MCF animals) stained for LANA served as negative controls. A formalin-fixed, paraffin-embedded cell pellet of BJ2586 cells (permanently OvHV-2 infected bovine large granulocyte lymphocyte cell line) served as a positive control for the anti-LANA antibody.[67] Sections from a cattle lymph node served as positive controls for cleaved caspase-3, Ki-67, CD3, CD20, Iba-1 and calprotectin, and sections from a rete mirabile of a non-MCF cattle as positive controls for α-SMA.

RNA in situ hybridization (RNA-ISH)

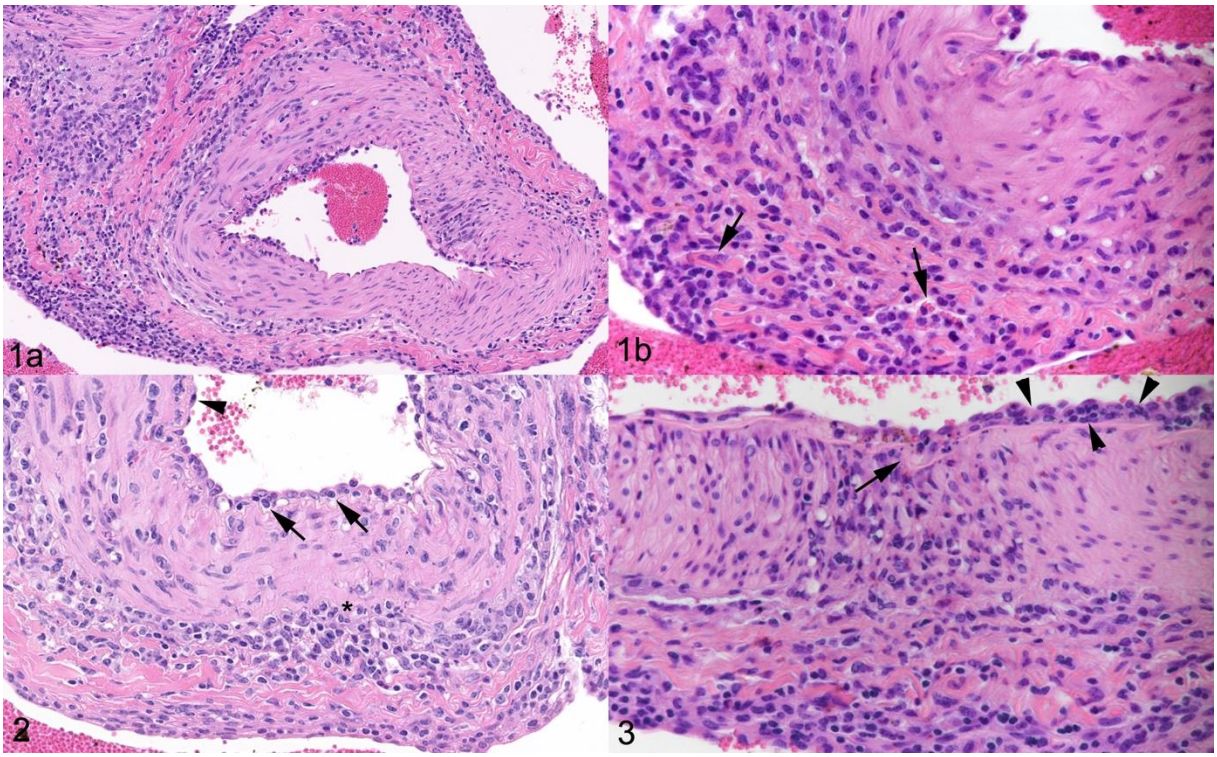
RNA ISH was performed using the RNAscope® ISH method (Advanced Cell Diagnostics (ACD, Newark, California) and the automated RNAscope® 2.5 Detection Reagent Kit (Brown) according to the manufacturer's protocol. All cases were first tested for the suitability of the tissue (RNA preservation and quality) with an oligoprobe for bos taurus peptidylprolyl isomerase B (PPIB). Those yielding good

PPIB signals were then subjected to RNA-ISH for Ov2.5 mRNA (coding for OvHV-2 viral IL-10; Genbank NC_007646.1).[18] Briefly, sections were heated to 60 °C for 1 h and subsequently deparaffinized. Permeabilization was achieved by incubating the section in pretreatment solution 1 (RNAscope® Hydrogen Peroxide) for 10 min at RT, followed by boiling in RNAscope® 1X Target Retrieval Reagents solution at 100 °C for 15 min and washing in distilled water and ethanol. After digestion with RNAscope® Protease Plus for 30 min at 40°C, sections were hybridized with the oligoprobes at 40°C in a humidity control tray for 2 h (HybEZ™ Oven, ACD Advanced Cell Diagnostics). Thereafter a serial amplification with different amplifying solutions (AMP1, AMP2, AMP3, AMP4: alternating 15 min and 30 min at 40 °C) was performed. Between each incubation step, slides were washed with washing buffer. They were subsequently incubated with AMP 5, AMP 6 and DAB at RT for 30 and 15 min respectively. Gill's hematoxylin served to counterstain the sections which were then dehydrated with graded alcohol and xylene and coverslipped. A formalin-fixed, paraffin-embedded pellet of OvHV-2-infected BJ2586 cells served as a positive control.

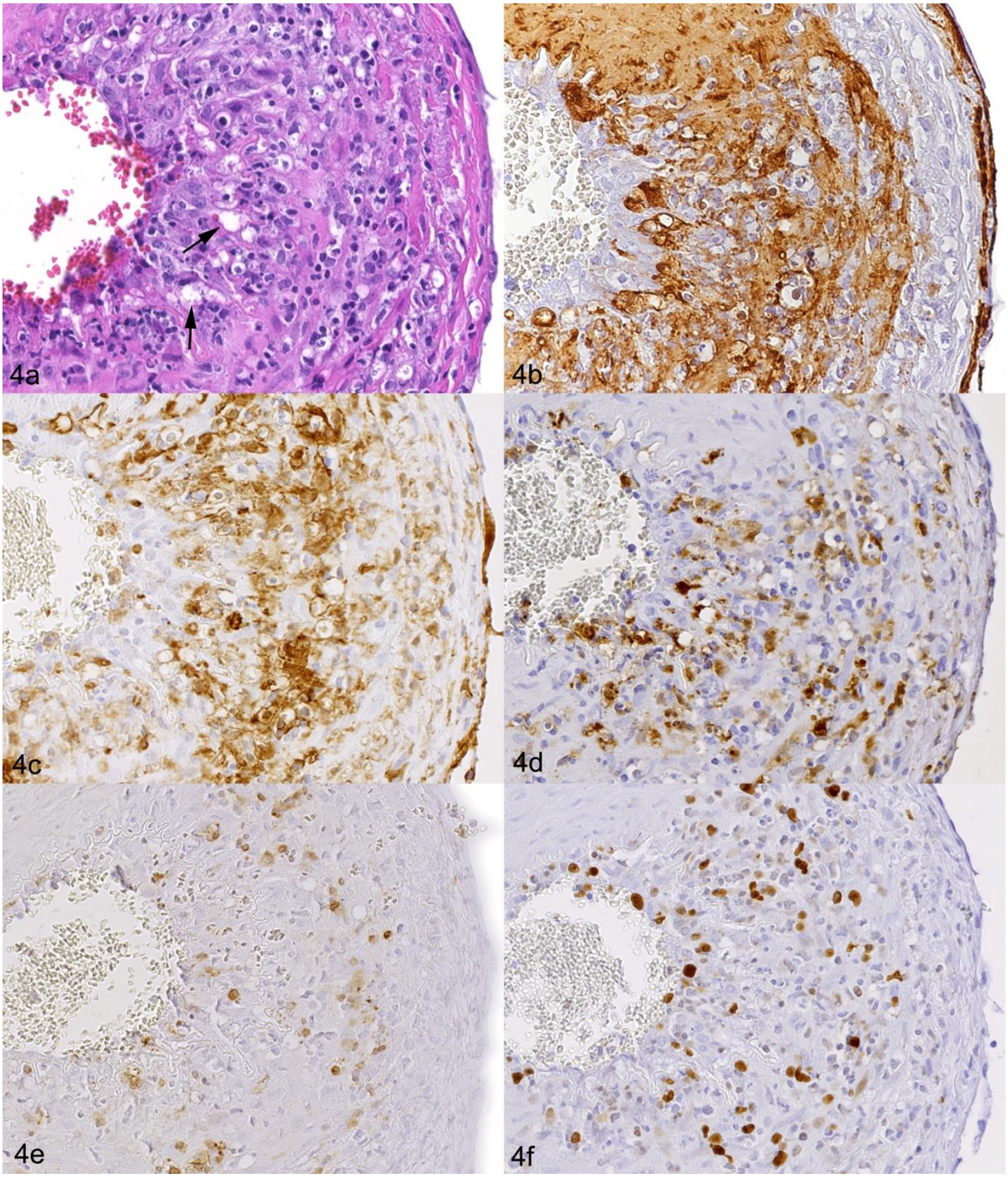
Results

MCF arteritis is variable in extent and severity, culminating in transmural inflammation with fibrinoid necrosis. In all cases, rete mirabile arteries consistently exhibited mononuclear infiltration of the tunica adventitia which formed a rim around each individual artery and also surrounded the vasa vasorum (Fig. 1). This was often accompanied by a focal to multifocal infiltration of the arterial wall that was frequently a continuum of the adventitial infiltrate (Fig. 1). In addition, affected arteries often

exhibited activated endothelial cells and evidence of leukocyte recruitment from the vessel lumen, represented by leukocytes that are located immediately beneath the endothelium (Figs. 2, 3); from there, the infiltrate appeared to often stretch into the tunica media (Fig. 3).



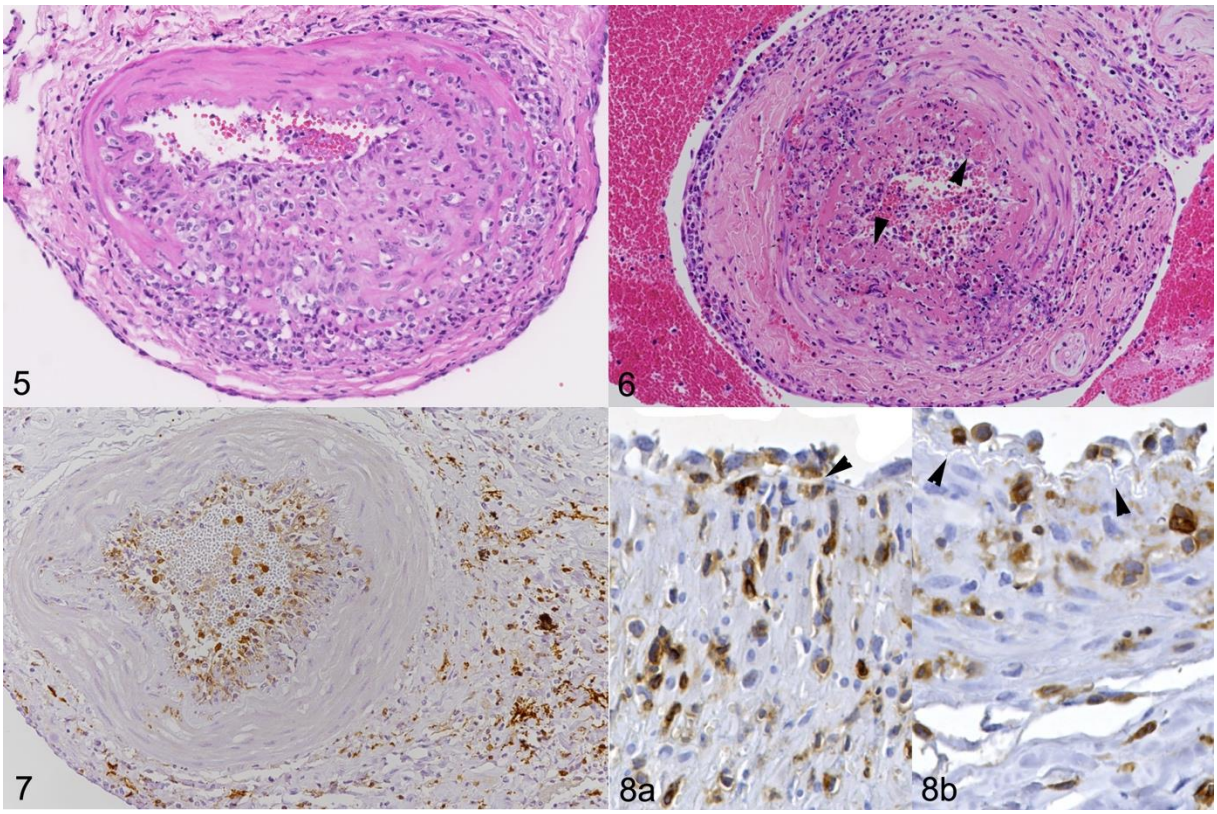
Some vessels also exhibited segmental transmural infiltrations (Figs. 3, 4).



The infiltrates in the media led to focal thickening of the wall (Fig. 5) and were sometimes associated with media disruption and/or degeneration of smooth muscle cells (SMC; Figs. 4a,b and 5). In a few cases (6/36; 16.7%) individual affected vessels exhibited necrosis of the intima, sometimes stretching through to the adventitia, with replacement of the tissue by amorphous hypereosinophilic material that contained cellular debris and pyknotic nuclei (Fig. 6). In all cases, a proportion of infiltrating leukocytes within arterial walls and the adventitia were found to undergo apoptosis (Fig. 7).

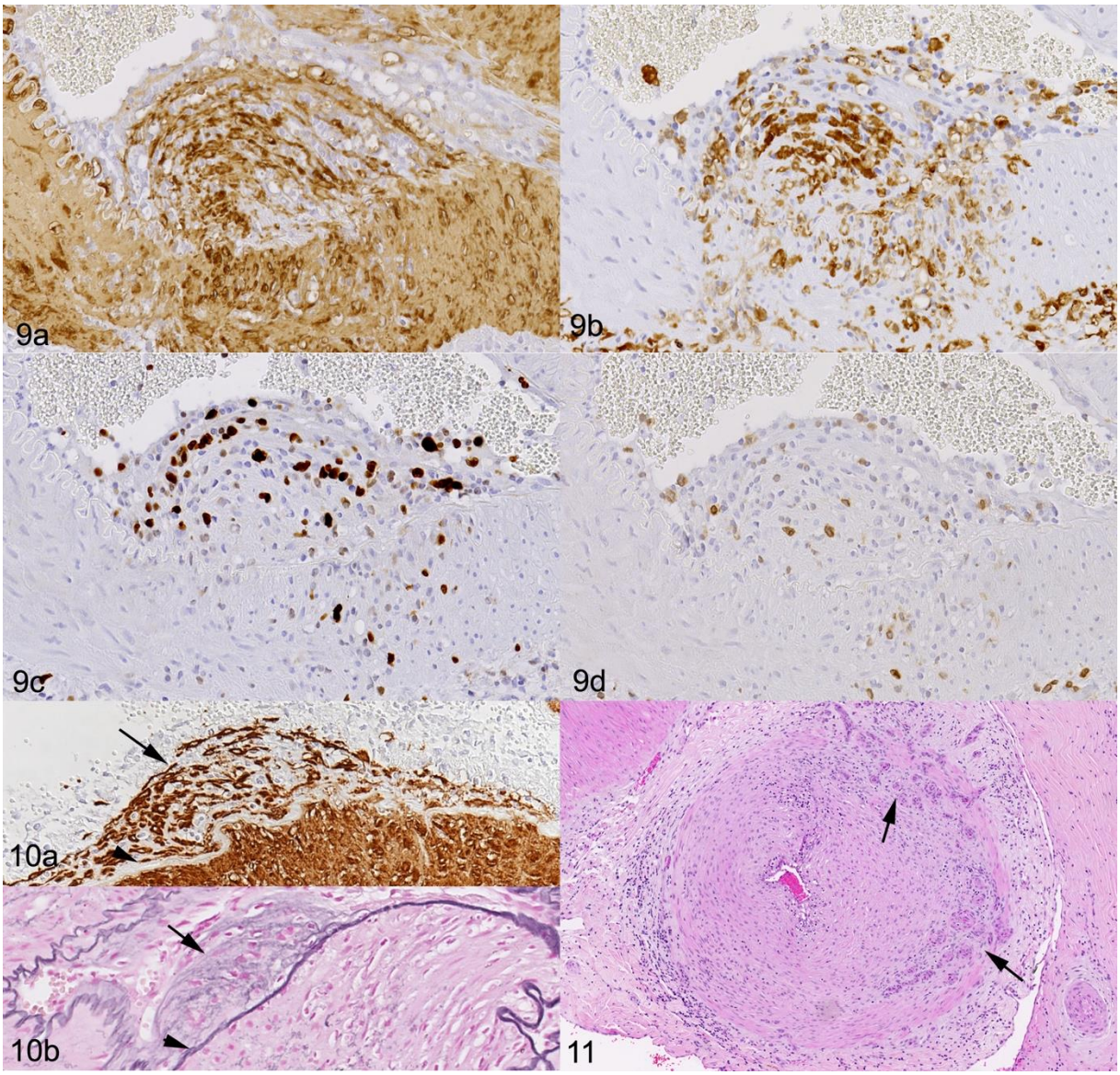
The described features were observed in the rete mirabile of all examined species (cattle, buffalo, bison). Differences were only seen in the extent, frequency and severity of the changes, both between individual animals and within the same animal.

Macrophages dominate the arteritis. In all cases and all affected vessels, macrophages (Iba-1+) and, often in lesser proportions, T cells (CD3+) were the principal infiltrating cells (Fig. 4c-e); B cells (CD20+) were rare and found scattered throughout the inflammatory infiltrates. Both T cells and macrophages appeared to be freshly recruited from the blood into the vascular wall (Fig. 8). For macrophages, the latter was proven as a proportion of cells in the infiltrate stained positive for calprotectin (Figs. 4d, 8a), a marker of monocytes and recently blood-derived macrophages.[14] However, a large proportion of cells within the wall infiltrates was found to proliferate, as shown by the abundance of Ki67-positive cells (Fig. 4f).



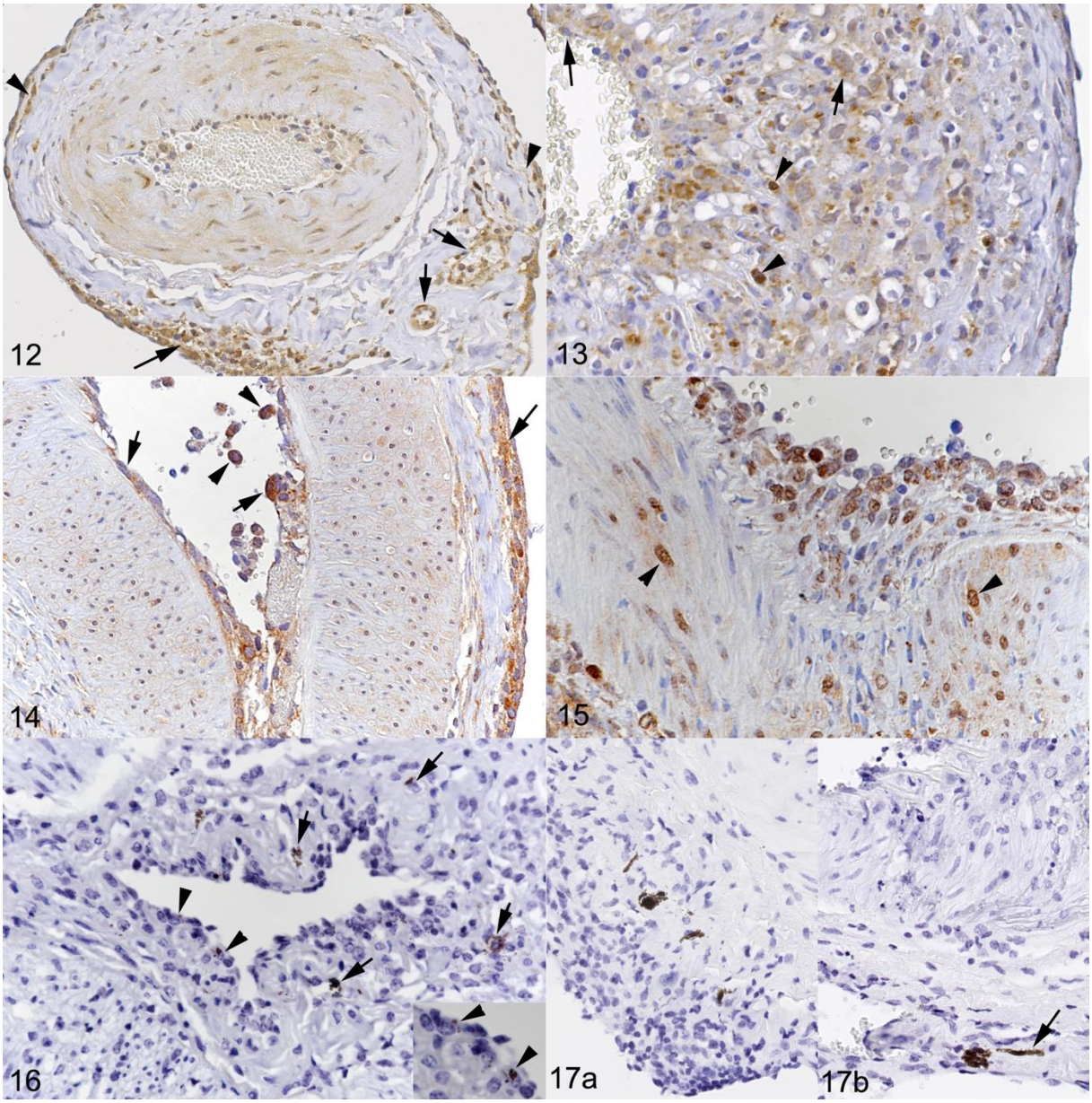
Chronic arterial lesions are characterised by muscular hypertrophy and intimal hyperplasia. In some affected vessels, thickening of the wall was not only due to the inflammatory infiltrates (Fig. 5), but also due to overall thickening of the muscle layer. Others showed a focal, cushion-like thickening of the intima, leading to luminal protrusion and narrowing of the vessel lumen (Fig. 9). These cushions were located between the endothelial cell layer and the tunica elastica interna and were comprised of loosely arranged SMC (Figs. 9a and 10a). Embedded between the latter were macrophages (Fig. 9b), several of which appeared to proliferate (Fig. 9c), and a few intermingled T cells (Fig. 9d). Within these areas, the internal elastic lamina appeared split and dissociated (Fig. 10). Occasionally, the arterial lumen was completely occluded by the intimal hyperplasia. Also, a perivascular rim of fibrosis and hyperplasia of vasa vasorum suggesting neoangiogenesis, was sometimes seen (Fig. 11). The most consistent and intense proliferative changes were observed in three animals where all rete mirabile arteries exhibited chronic changes; the vessels

only exhibited minimal inflammatory infiltration. Different from other cases where the variety of changes suggested that not all arteries had been affected at the same time, these animals had likely passed the peak of the disease at an earlier stage and now only exhibited the remnants of the original inflammatory processes.[42,43,59]



MCF arteritis is associated with the presence of oLANA protein and vIL-10 transcription. Both oLANA and ovIL-10 mRNA expression were detected in the rete mirabile arteries, with and without inflammatory changes. oLANA was widespread. It was seen in leukocytes, within the vessel lumen, rolling along the endothelium and in the inflammatory infiltrates in the arterial walls, and was present in endothelial cells

and SMC, mainly within the cytoplasm, but occasionally also in the nucleus (Figs. 12-15). In the chronic cases with scant inflammation, faint staining was seen in scattered endothelial cells and SMC in the tunica media. In the adventitia, oLANA was seen in infiltrating cells, in endothelial cells of the vasa vasorum, and in fibroblasts (Figs. 12, 14). ovIL-10 mRNA showed the same expression pattern, though the number of positive cells was far lower (Figs. 16, 17). It was also detected in occasional inflammatory cells and fibroblasts in the adventitia (Fig. 17).



Discussion

MCF is a sporadic disease of ruminants with high mortality. One of its pathological hallmarks is a vasculitis, which presents predominantly as a mononuclear arteritis, with variable additional features, such as necrosis, intimal hyperplasia and obliterative changes.[30,59,68]

In sheep, the natural hosts of OvHV-2, the MCFV that is also the focus of the present study, the virus targets CD4+ and CD8+ T cells,[36,58] where it predominantly persists in its latent form.[67] Animals with MCF also carry OvHV-2 in blood lymphocytes, however, these are not only latently,[35] but at least in a proportion also productively infected.[67] Productively infected lymphocytes, and specifically cytotoxic CD8+ T cells, have also been detected in and around MCF vasculitis,[41,49,61] and there is evidence of abortive infection of perivascular fibroblasts associated with the vasculitis.[41]

The present detailed study on rete mirabile arteries in MCF affected animals aimed to shed more light on the pathogenesis of MCF arteritis and laid the focus on infiltrating cells and the presence of OvHV-2 gene products that are expressed during latency, viral (ov)IL-10 mRNA and oLANA,[67] in affected vessels.

A relevant role for macrophages in MCF arteritis. Previous studies have shown that, besides lymphocytes, macrophages and, to a low extent, neutrophils and plasma cells are present in MCF vasculitis.[29,30,39] Overall, macrophages seemed not to represent a relevant leukocyte population in the infiltrates.[1,61] The results of the present study suggest the opposite, showing that macrophages are consistently abundant in MCF vasculitis, outnumbering or at least equalling the T cell population; other leukocytes were not present in relevant numbers. This provides evidence that macrophages are, beside CD8+ T cells, key cells in MCF vasculitis. Both infiltrating macrophages and T cells appear to proliferate locally, as numerous infiltrating cells

were Ki67-positive. This proliferation likely reflects an autocrine effect of the cytokine and growth factor release by the leukocytes, and thereby a consequence of the ongoing inflammatory stimulus. At the same time, we found evidence of ongoing monocyte recruitment into the lesions, as a proportion of infiltrating macrophages was found to express calprotectin, a marker of monocytes and recently blood derived macrophages.[14]

The leukocyte infiltrates in the media were sometimes accompanied by media disruption and/or degeneration of SMC. Also these can be the result of macrophage infiltration, as activated macorphages release reactive oxygen species (ROS) and matrix metalloproteinases (MMPs), in particular MMP-2 and -9, that will destruct the media and digest the internal elastic lamina.[57,60]

We have not investigated the macrophage subpopulations, ie. M1 and M2 macrophages, in MCF vasculitis, which could provide further information on the pathogenic processes involved. However, in human giant cell arteritis, for exampe, both occur, and the characteristics of MCF arteritis suggest that this is also the case here.[14,60]

Arteritis in the rete mirabile is an "outside-in" vasculitis followed by "inside-out" infiltration. Medium sized and larger arteries, like those in the rete mirabile, are multilayered vessels that are surrounded by the adventitia which contains a delicate microvasculature, the vasa vasorum. The latter supply oxygen and nutrients to the vessel wall and are the first site of response to arterial injury, providing access for inflammatory cells. Due to this vessel architecture, two different pathways of arterial inflammation have been suggested for arteritis in humans, a traditional concept known as "inside-out" and the newer paradigm of an "outside-in" pathway.[33,38]

Similar to other authors who reported on the features of the inflammatory processes in the rete mirabile in MCF,[30] we observed that the infiltration is generally most intense in or even restricted to the tunica adventitia of the arteries, with only focal and frequently less substantial infiltration of the tunica media; the latter often appeared to be a continuum of the adventitial infiltrate. This provides strong evidence that the inflammatory cells are recruited to the vessel through the vasa vasorum and speaks for an “outside-in” pathway.[33,38] In humans, under inflammatory conditions, in particular macrophages are recruited through this microvasculature; this is also suggested in our study, considering that the infiltrating cells were in the majority macrophages.[14] The “inside-out” inflammatory process, however, is triggered by injured endothelial cells. These express/upregulate surface adhesion molecules and inflammatory mediators that participate in monocyte homing to the endothelium and eventual transmigration into the media.[33] In the case of MCF arteritis it appears likely that monocytes are recruited into the adventitia from the blood via the vasa vasorum, differentiate into macrophages, become activated and secrete inflammatory cytokines which results in further inflammatory cell recruitment. With subsequent infiltration of the media mediators released from the macrophages will lead to activation of the arterial endothelial cells which could then trigger the inside-out inflammatory process that we also observed in a proportion of affected vessels. Indeed, in these vessels the endothelial cells appeared activated and intravascular leukocytes were found attached to the endothelium, extravasating, and within the media.

Arteritis in the rete mirabile can culminate in necrotizing inflammation or progress towards arterial remodeling. In addition to the inflammatory infiltration, arteries were occasionally found to exhibit more pronounced changes. In some cases where the arteries showed different degrees of inflammatory infiltration, individual affected

vessels exhibited extensive damage to the wall, represented by circumferential accumulation of fibrin at the intima-medial junction, overlain by large numbers of intact and degenerating/ necrotic inflammatory cells at the luminal surface. Among the infiltrating cells were some, mainly degenerate, neutrophils. The MCF literature reports such fulminant changes as a finding of variable frequency and has interpreted these as fibrinoid necrotising vasculitis.[1,30,37,59] Since the latter is generally seen as a consequence of antigen-antibody complex deposition, this pathogenesis has indeed been considered for MCF vasculitis.[30] However, there has neither been convincing evidence of virus-specific immune complexes in vessel walls in MCF,[48] nor have ultrastructural studies of MCF vasculitis found any evidence of immune complex deposition in the lesions.[30,42] Instead, it is possible that the necrosis is a secondary event, resulting, for example, from the release of reactive oxygen species by infiltrating macrophages,[60] or due to ischemia as a result of an increase in wall thickness due to the inflammatory infiltrate, and insufficient oxygen supply through the vasa vasorum.[38]

We also observed chronic proliferative arterial changes in some affected arteries, features that have been reported in MCF cases with prolonged clinical disease or after clinical recovery.[42,43,59] In a few animals, these represented the only type of vascular lesion, in others they were present in some affected vessels, usually in the form of focal intimal hyperplasia. The proliferative changes were associated with a variable degree of focal or circumferential luminal narrowing, as a consequence of subendothelial accumulations of spindle-shaped, in the majority α-SMA positive cells. The latter were associated with disarrangement/splitting of the internal elastic lamina, and the spindle-shaped cells were intermingled with macrophages and a few T cells that all seemed to proliferate, as shown by the extensive Ki-67 expression. Similar subendothelial accumulations of spindle-shaped cells have previously been reported

in recovered MCF cases.[42,43]. In the literature, these accumulations are also referred to as neointimal proliferation as they are located immediately beneath the endothelium and luminal to the internal elastic lamina. The cells represent SMC of the tunica media that have migrated to the intima, or myofibroblasts originating from adventitial fibroblasts that have undergone a phenotypic switch acquiring α-SMA expression, and have migrated to the intima.[33,38]

The observed chronic changes are consistent with vascular remodeling processes and have several features in common with lesions observed in chronic arteritis in humans, for example giant cell arteritis (GCA).[57] Similar to MCF arteritis, GCA is associated with the recruitment of CD8+ T cells and monocytes into the arterial wall due to so far unknown pathogenic processes. The following scenario is suggested: Through the release of IFN-γ the T cells can recruit the monocytes, and they can induce the observed apoptosis of cells in the media via cytotoxic molecules such as granzyme B and perforin.[40] Monocytes differentiate into macrophages which release cytokines that perpetuate the inflammatory response, toxic mediators such as reactive oxygen species, and MMPs that further destroy the media. Also, cytokine-activated endothelial cells can recruit more inflammatory cells into the vascular wall. Then, growth factors released by macrophages and SMC come into action; PDGF can induce proliferation and migration of SMC, leading to the observed intimal hyperplasia, and VEGF can induce neoangiogenesis.[57] We have evidence of the latter as we saw local hyperplasia of vasa vasorum in a few arteries, a feature also known to occur when the artery cannot be sufficiently supplied from the luminal surface, eg. with increased thickness of the wall.[38] Interestingly, Epstein Barr virus infection has been associated with pulmonary vascular remodelling in human idiopathic pulmonary fibrosis, and a similar effect was seen in CD1 mice infected with murine γ-herpesvirus (MHV)-68.[9]

Broad expression of oLANA and ovIL-10 mRNA in MCF vasculitis suggests a role of latent virus in the pathogenesis. Affected arteries exhibited variable, but consistent OvHV-2 oLANA expression in endothelial cells and SMC in the vascular wall and in both intravascular and infiltrating lymphocyes and macrophages. This indicates a vascular target cell spectrum in MCF that is broader than anticipated based on previous studies, though these detected lytic infection which may indeed be restricted to CD8+ T cells.[40,41,49,61] oLANA was also found in occasional endothelial cells in vessels without inflammatory changes, indicating more widespread viral latency in the endothelium. LANA is a nuclear protein that is encoded by ORF73 and expressed during viral latency.[18,67] The Kaposi's Sarcoma Herpesvirus (KSV) LANA is a homologue with sequence similarity to OvHV-2 oLANA. KSHV LANA is a multifunctional protein that is responsible for the persistence of the viral episome in mammalian cells. It also enhances survival and proliferation of infected cells, and seems to have an immunomodulating function, damping down the IFN response and slowing down MHC I recognition.[34,70,71] It is therefore likely, by inference, that oLANA has similar functions during OvHV-2 infection. A role for the AlHV-1 LANA in MCF has been shown, at least with regards to the proliferation of T cells.[47] Unexpectedly, we observed more intense cytoplasmic than nuclear staining for OvHV-2 LANA which suggests that, similar to KSV LANA, the protein has cytoplasmic isoforms. Previous studies have shown that cytoplasmic KSHV LANA may be involved in viral activation, with transition from the latent to the lytic cycle.[34,71] This would be consistent with a recent RNA-ISH study that detected lytic viral infection in lymphocytes in MCF vasculitis.[49] Similarly, OvHV-2 oLANA transcription has previously been reported in tissues of bisons with MCF, though alongside ORF25 transcription which showed that lytic infection was also taking place.[11]

We also examined the rete mirabile for the transcription of ov2.5, a latency associated gene that encodes for ovIL-10, a protein similar to ovine interleukin IL-10 that has retained its function, such as the induction of mast cell proliferation and the inhibition of macrophage function.[22] Using RNA-ISH, we found ovIL-10 transcription in a similar set of cells in MCF vasculitis as viral LANA, i.e. vascular endothelial cells and myofibroblasts/SMC as well as infiltrating leukocytes (macrophages and lymphocytes), the latter predominantly in the tunica adventitia. We also saw it occasionally in endothelial cells and adventitial fibroblasts of unaffected arteries. Viral interleukines, acquired during coevolution with their host species and encoding for orthologues of cellular interleukins, provide a viral strategy to deregulate the host immune system.[46,62] ovIL-10 has a direct immunosuppressive effect on macrophages, inhibiting the release of proinflammatory mediators (Jayawardane et al. 2008).[22] The principal action of cellular IL-10 is to modulate immune responses and to protect from their damage.[55] By hijacking cIL-10, OvHV-2 might evade an immune response; its presence in MCF vasculitis could suggest that it is also associated with modulation of macrophage function that might be crucial for their interaction with equally infected (CD8+) T cells.

Latent infection and thereby oLANA and ovIL-10 expression were not associated with degenerative changes in the cells, though occasional apoptotic cells appeared to be oLANA-positive. The same is apparently true for the cells undergoing lytic or abortive infection suggesting that if the virus induces functional or phenotypic changes in infected cells, it does this without cytopathic effects. The immune response to the virus may play a role here; this is suggested by the fact that MHV-68 can infect vascular SMC and cause arteritis in immunodeficient mice (Weck et al., 1997).

The key question regarding the pathogenesis of MCF vasculitis is therefore, what sets off the inflammatory cell recruitment in the first place. Whether this is the

activation of endothelial cells and circulating T cells/monocytes by (latent) OvHV-2 remains to be elucidated. Also, the effect of OvHV-2 infection on other adventitial cells, such as fibrobasts or medial SMC which were both found to be latently infected, warrants further investigations. [41] Adventitial fibroblasts are activated under pathological conditions and undergo phenotypic changes which include proliferation, differentiation into myofibroblasts and migration to the intima, increased production of extracellular matrix proteins, and release of factors (i.e. reactive oxygen species) that directly or indirectly affect vascular function and structure. In this context, α-SMA expression is triggered by a complex microenvironment that enhances fibroblast differentiation into a myofibroblast phenotype where TGF-β induces this phenotypic switch.[16]

The literature has provided strong evidence that the development of MCF is a consequence of failure of the immune system to maintain a balance between restricted growth and uncoordinated multiplication of infected cells which have the phenotype of T cells with unrestricted killer activity. However, our results suggest that T cells are not the only leukocytes that are latently infected, and that monocytes and locally proliferating macrophages are at least as important for the vasculitis. What remains to be identified is the initial trigger/insult that leads to their activation. There is at least morphological *in situ* evidence that latently infected, acvitated endothelial cells play a role in this. Activated monocytes/macrophages would then release the necessary pro-inflammatory mediators and, eventually, induce the characteristic vascular changes.

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Figure Legends

Figures 1-3. MCF, case no 15, rete mirabile arteries. HE stains. **Figure 1. a**. Moderate mononuclear infiltration of the adventitia, forming a rim around the artery. **b**. The higher magnification shows the adventitial infiltrate with embedded vasa vasorum (arrows). **Figure 2**. The inflammatory infiltrate in the adventitia expands into the media (*). Endothelial cells are activated (arrowhead), and there are individual leukocytes immediately beneath the endothelium (arrows), indicating recruitment from the arterial lumen. **Figure 3**. The affected artery exhibits areas with a layer of leukocytes immediately beneath the endothelium (arrowheads) from which the infiltrate appears to stretch focally into the underlying media. This is associated with focal destruction of the internal elastic lamina (arrow).

Figure 4. MCF, case no 18, rete mirabile artery. **a**. Transmural infiltration with multifocal vacuolation of the media (arrow). HE stain. **b**. Within areas of media vacuolation, the smooth muscle cell (α-SMA+) arrangement is disrupted. **c**. The inflammatory infiltrate is dominated by Iba-1 positive macrophages. **d**. A moderate proportion of the infiltrating macrophages are recently blood derived, as indicated by their calprotectin expression. **e**. T cells (CD3+) are far less numerous. **f**. Staining for Ki-67 shows that a substantial proportion of infiltrating cells is proliferating. Immunohistology, hematoxylin counterstain (b-f).

Figures 5-8. MCF cases, rete mirabile arteries. **Figure 5**. Case no 1. The infiltration of the media is associated with focal thickening of the arterial wall. The media appears disrupted by the infiltrate. HE stain. **Figure 6**. Case no 7. Affected artery with circular intimal necrosis that stretches into the media, with replacement of the tissue

by amorphous hypereosinophilic material (arrowheads) that contains cellular debris and pyknotic nuclei. HE stain. **Figure 7**. Case no 7. A proportion of infiltrating cells in the arterial wall and the adventitia is undergoing apoptosis, as shown by staining for cleaved caspase 3. Immunohistology, hematoxylin counterstain. **Figure 8**. Case no 11. Macrophages and T cells are recruited from the blood into the vessel wall. Staining for calprotectin (**a**) confirms the macrophages as recently blood derived. Both macrophages (**a**) and T cells (CD3+) (**b**) are seen attached to and immediately beneath endothelial cells and migrate beyond the internal elastic lamina (arrowheads). Immunohistology, hematoxylin counterstain.

Figures 9-11. MCF cases, rete mirabile arteries. **Figure 9**. Case no 21. Artery with focal, cushion-like intimal thickening due to (**a**) α-SMA-positive smooth muscle cells/myofibroblasts with (**b**) infiltrating macrophages (Iba-1+) which (**c**) appear to proliferate (Ki-67+). **d**. T cells (CD3+) are a minority in the infiltrate. Immunohistology, hematoxylin counterstain. **Figure 10**. Case no 21. Within areas of focal cushion-like thickening (arrows), the internal elastic lamina (arrowheads) appeared split. **(a)** Immunohistology for α-SMA, hematoxylin counterstain; **(b)** van Gieson stain. **Figure 11**. Case no 35. Artery with circular thickening of the wall, severe narrowing of the arterial lumen, and hyperplasia of vasa vasorum (arrows). HE stain.

Figures 12-17. MCF cases, rete mirabile arteries. **Figures 12-15**. OvHV-2 LANA expression. Immunohistology, hematoxylin counterstain. **Figure 12**. Case no 11. Widespread expression of OvHV-2 LANA in inflammatory cells in the adventitia (long arrow), in endothelial cells of the vasa vasorum (short arrows), and individual fibroblasts (arrowheads). Leukocytes within the vessel lumen, endothelial cells and smooth muscle cells in the arterial wall exhibit weak staining. **Figure 13**. Case no 18.

Artery with transmural infiltration. oLANA is detected in abundant infiltrating cells, mainly within the cytoplasm, but occasionally also in the nucleus (arrowheads) of cells that appear to be apoptotic, in smooth muscle cells in the media (short arrow) and, weakly, in endothelial cells (long arrow). **Figure 14**. Case no 11. Artery with onset of intimal infiltration, showing oLANA in leukocytes in the vessel lumen (arrowheads) and endothelial cells (short arrows). There is also weak staining in the media. Leukocytes in the adventitial infiltrate are also oLANA-positive (arrow). **Figure 15**. Case no 16. Arterial wall with intimal mononuclear infiltration stretching into the media. oLANA expression is detected in leukocytes in the vessel lumen and in the infiltrate. Smooth muscle cells exhibit both nuclear and weak cytoplasmic expression (arrowheads). **Figures 16, 17**. OvHV-2 IL10 transcription. RNA-ISH, hematoxylin counterstain. **Figure 16**. Case no 30. ovIL-10 signal is observed in leukocytes in the intimal and media infiltrate (arrows) and in endothelial cells (arrowheads). **Figure 16**. Case no 1. **a.** The infiltrate in the adventitia contains cells with ovIL-10 signal. **b**. There are also fibroblasts that express ovIL-10 mRNA (arrows).

Supplemental Table S1. Cases of malignant catarrhal fever included into the study. OvHV-2 infection was confirmed by qPCR on EDTA blood samples or tissues, and/or by RNA-ISH for OvHV-2 Ov2.5. Also, OvHV-1 LANA protein expression was detected within the arteries of the rete mirabile in all cases.

Case No	Species	Breed	Sex	Age (mo/y)
1[a, b]	Cattle	Brown Swiss	F	3 y
2[a, b]	Cattle	Red Holstein	F	7 mo
3 [a, b]	Cattle	Red Holstein	F	4 y
4 [a, b]	Cattle	Brown Swiss	F	1.5 y
5 [a, b]	Cattle	Brown Swiss	F	2 y
6 [a, b]	Cattle	Dexter	M	6 mo
7 [a, b]	Cattle	Red Holstein	F	3 y
8 [a, b]	Cattle	Red Holstein	F	4.5 y
9 [a, b]	Cattle	Holstein Friesian	F	6 mo
10 [a, b]	Cattle	Red Holstein	F	3.5 y
11[a, b]	Cattle	Holstein Friesian	F	3 y
12[a]	Cattle	Brown Swiss	F	9 mo
13[a]	Cattle	Brown Swiss	F	4 y
14[a]	Cattle	Brown Swiss	F	7.5 mo
15[a]	Cattle	Simmental	F	6 y
16[a]	Cattle	Simmental	F	1 y
17[a]	Buffalo	Water Buffalo	F	2 y
18[a]	Buffalo	Water Buffalo	F	12 y
19[a]	Cattle	Eringer	F	1.5 y

20[a]	Cattle	Holstein Friesian	F	3.5 y
21[a]	Buffalo	Amazone buffalo	F	np
22[a]	Cattle	Brown Swiss	F	9 y
23[a]	Buffalo	Water buffalo	F	2.5 y
24[a]	Bison	np	F	np
25[a]	Cattle	Brown Swiss	F	4 y
26[a]	Buffalo	Water buffalo	F	1.5 y
27[a]	Buffalo	Water buffalo	F	2 y
28[a]	Bison	np	M	2.5 y
29[b]	Cattle	Red Holstein	F	1.5 y
30[b]	Cattle	Holstein Friesian	F	1 y
31[b]	Cattle	np	F	2 y
32[b]	Cattle	Red Holstein	F	14 mo
33[b]	Cattle	np	F	1 y
34[b]	Cattle	np	F	1.5 y
35	Buffalo	Water buffalo	M	np
36	Cattle	Holstein Friesian	F	1 y

F, female; M, male; y, years; mo, months; np – not provided

[a] OvHV-2 infection confirmed by qPCR on EDTA blood or formalin-fixed, paraffin embedded tissue specimens

[b] RNA-ISH for OvHV-2 Ov2.5 positive

Supplemental Table S2. Antibodies, antigen retrieval and detection methods used in immunohistology

Antigen	Antibody (clone)	Source	Dilution (in PBS)	Pretreatment	Detection method
Calprotectin	Mouse mAb (MAC 387)	Abcam	1:100	EDTA	EnVision Mouse
Cleaved caspase 3	Rabbit mAb (D175)	Cell Signalling	1:100	EDTA	EnVision Rabbit
CD3[a]	Mouse mAb (F.7.2.38)	Dako/Agilent	1: 100	EDTA	EnVision Mouse
CD20	Rabbit pAb	Thermo Scientific	1:1,000	ND	EnVision Rabbit
Iba-1	Rabbit pAb	Wako	1:750	Citrate	EnVision Rabbit
Ki67	Mouse mAb (MIB-1)	Dako/Agilent	1:50	EDTA	Dako REAL
LANA	Rabbit pAb	Custom made	1:250	Citrate	EnVision Rabbit
α-SMA	Mouse mAb (1A4)	Dako/Agilent	1:400	ND	Dako REAL

Iba-1, ionized calcium binding adaptor molecule 1; α-SMA, α-smooth muscle actin; LANA, latency associated nuclear antigen; pAb, polyclonal antibody; mAb, monoclonal antibody; HRP, horseradish peroxidase; ND, not done.

EDTA, 20 min incubation in EDTA buffer (pH9; Dako/Agilent) in a pressure cooker at 98 °C. Citrate, 20 min incubation in citrate buffer (pH 6; Dako/Agilent) in a pressure cooker at 98 °C.

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Gracias

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